ABBS

Review

Phoenix rising: gene therapy makes a comeback

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Despite the first application of gene therapy in 1990, gene therapy has until recently failed to meet the huge expectations set forth by researchers, clinicians, and patients, thus dampening enthusiasm for an imminent cure for many life-threatening genetic diseases. Nonetheless, in recent years we have witnessed a strong comeback for gene therapy, with clinical successes in young and adult subjects suffering from inherited forms of blindness or from X-linked severe combined immunodeficiency disease. In this review, various gene therapy vectors progressing into clinical development and pivotal advances in gene therapy trials will be discussed.

Introduction

Long before the discovery of specific genes that cause disease and years prior to the publication of the DNA structure by Dr James D. Watson and Dr Francis Crick [1], Dr Clyde E. Keeler predicted in 1947 that gene therapy would be 'a therapeutic technique' 'achieving permanent correction of hereditary diseases' [2]. Several years later in 1972, Dr Theodore Friedmann and Dr Richard Roblin predicted that 'gene therapy may ameliorate some human genetic diseases in the future' [3]. True to these predictions, gene therapy vectors have been the subject of extensive development over the last 40 years in efforts to insert or replace therapeutic genes in target cells of affected tissues and cure genetic diseases. Proof-of-principle for gene therapy became a reality in 1990, when 4-year-old Ashanti DeSilva, suffering from adenosine deaminase severe combined immunodeficiency (ADA SCID), was a subject of an *ex vivo* gene therapy trial [4,5]. A subset of her T cells was removed, treated *ex vivo* with a gammaretrovirus expressing the ADA gene, and the gene-corrected T cells reintroduced into her circulation [5]. This process essentially reconstituted her immune system with gene-corrected T cells. Ashanti is now a healthy 27-year-old.

Delivery and expression of a therapeutic gene in an affected tissue or organ holds great promise for the treatment of various life-threatening acquired and inherited diseases that affect humans. Gene therapy is a simple concept: delivery of DNA (or RNA) to the affected somatic cells of a specific tissue or organ will repair the defect, restore normal function and cure the subject of the specific disease phenotype. Almost 20 years ago cystic fibrosis (CF) [6] airway disease was characterized as an excellent candidate for gene therapy [7] on the basis that it was a monogenic disease [8,9] and the level of gene expression necessary for correction of the disease phenotype was low (~5%) [10].

Gene Therapy Vectors

The gene transfer tools that have been developed for the delivery of genes to various targets are either viral-based or non-viral-based (**Table 1**). Viral-based vectors exploit the ability of a virus to effectively transfer its genetic cargo into the target cell. For most gene therapy protocols designed for lifelong genetic diseases, re-administration of the virus-based gene transfer vector will be warranted. However, the majority of virus-based vectors induce potent humoral immune responses that will diminish the effectiveness of vector re-administration [11–13]. Additionally, pre-existing immunity to a specific virus-based vector due to a previous infection with the wild-type virus or an earlier vector administration will also limit effective gene transfer. Indeed, in recent years this has been shown to be case in a number of preclinical and clinical models of gene transfer [11]. In contrast, non-viral vectors consist of lipids, peptides, carbohydrates, or nanoparticles that fuse with the cell membrane and release the therapeutic DNA into the cell cytoplasm and induce low, if any, immune responses. Below, representative gene therapeutics from both categories are discussed in detail.

Adenovirus-based vectors
Adenoviruses (AdV) are non-enveloped viruses with a linear double-stranded DNA genome and belong to the Adenoviridae family. Adenoviruses are common pathogens

Table 1 Comparison of the most commonly used vectors for gene therapy

	Adenovirus vector	Adeno-associated virus vector	Lentivirus vector	Retrovirus vector	Liposomes
Tropism	Dividing and non-dividing cells	Dividing and non-dividing cells	Dividing and non-dividing cells	Dividing cells	Dividing and non-dividing cells
Host genome	No integration	No integration	Integration	Integration	No integration
Transgene expression	Transient	Stable	Stable	Stable	Transient
Packaging capacity	~8 kb	~5 kb	~8 kb	~8 kb	>20 kb
Advantages	Large packaging capacity; high production yields	High production yields; low immunogenicity; long-term expression	Large packaging capacity; long-term expression	Large packaging capacity; long-term expression	Low immunogenicity
Disadvantages	High immunogenicity; transient expression	Small packaging capacity	Insertional mutagenesis	High risk of insertional mutagenesis	Transient expression

of the respiratory tract accounting for ~10% of upper respiratory infections in young children and older adults [14]. Given their large cloning capacity (~8 kb) [15], they have been developed as gene therapy vectors for various genetic diseases including CF [16]. Although there are ~50 serotypes of AdV, vectors based on serotypes 2 and 5, both members of subgroup C [17], have been extensively reported to efficiently transfer transgenes to many cell types *in vitro* and to various tissues or organs *in vivo* [18]. AdV vectors can be produced at high titers [19] with minimal risk of contamination from replication-competent virus. However, as AdV vectors are non-integrative and non-replicating, they can only confer transient gene expression, the duration of which is dependent on the proliferation state of the target cell [20,21]. AdVs have four early genes (E1−4), which encode polypeptides responsible for viral and cellular gene expression, viral replication, repression of major histocompatibility complex responses and inhibition of cellular apoptosis [21]. In first-generation AdV vectors, the early region (E1A/B), containing the transcriptional regulatory genes essential for viral replication, was replaced with an expression cassette for a transgene [22,21]. These AdV vectors are propagated in E1-trans-complementing cell lines such as HEK293 [21]. The administration of first-generation AdV vectors in animals and in subjects in gene therapy clinical trials was found to elicit high inflammatory and immune responses. These responses were T cell-mediated and directed against both the vector and the transgene product, resulting in the elimination of transduced cells and compromising the duration of gene expression [23,24]. In second generation AdV vectors, in addition to the E1 region, the E2, E3, or E4 regions were either deleted or disabled [25]. As a consequence, these AdV vectors exhibited lower levels of viral

protein expression shown to be responsible for evoking host immune and inflammatory responses. The gene transfer efficiency of second-generation AdV vectors was no different than that of the first-generation AdV vectors [25]. In an effort to improve both the duration of gene expression and safety, helper-dependent AdV vectors were developed. These vectors are devoid of all viral coding sequences, do not integrate into the host genome, and result in long-term gene expression *in vivo* [26].

In a seminal moment in the clinical translation of gene therapy, a serious adverse event occurred in a clinical trial for ornithine transcarbamylase deficiency with the death of 18-year-old Mr Jesse Gelsinger who was enrolled in the sixth cohort of the study that received the highest dose of the E1/E4-deleted AdV vector [27]. The patient suffered an acute and severe inflammatory response that resulted in his death almost 4 days after vector injection [28]. The unfortunate outcome of this trial severely dampened enthusiasm for gene therapy. In a positive way, however, this event [28] served to re-focus the attention of the field to the host immune responses against the viral capsid, as well as the expressed therapeutic transgene product, paving the way for future gene therapy successes.

Adeno-associated virus-based vectors

Adeno-associated virus (AAV) was discovered as a contaminant in an AdV vector stock preparation [29] and was found to require the presence of an AdV or herpesvirus to replicate *in vitro*. AAV is a non-enveloped single-stranded DNA virus that belongs to the Parvoviridae family. The cloning capacity of AAV is ~4.7 kb [30], which limits its development for genetic diseases that require the expression of large therapeutic transgenes, such as the CF gene (cystic fibrosis transmembrane conductance regulator,

CFTR [8]) for CF airway disease. AAV vectors can positively transduce dividing and non-dividing cells, an advantage for those organs in which <1% of cells are actively dividing (e.g. lung [31]). To date, AAV has not been associated with any known human disease and is considered a safe vector for use in humans. To overcome dependence on second-strand synthesis, self-complementary AAV (scAAV) vectors were developed [32]. The two main benefits of these vectors were the rapid onset of gene expression and the significantly higher (up to 600-fold [33]) levels of transgene expression compared with their single-stranded counterparts. However, the main disadvantage associated with the use of scAAV vectors is the further reduced cloning capacity to ∼2.2 kb [32]. In contrast to wild-type AAV, which integrates specifically into the q arm of chromosome 19 [34], AAV vectors (including scAAV) integrate randomly (raising the possibility of insertional mutagenesis), or remain as stable episomal DNA [35]. In recent years, it was demonstrated that AAV vectors preferentially integrated into active genes following systemic delivery to the mouse liver raising the potential for cancer development [36]. AAV vector-mediated integration however, is not as common as that observed for retroviruses. AAV vectors do not possess the machinery necessary to activate genes or cause breaks in host chromosomes [37]. In subsequent large and long-term liver-directed gene transfer studies in mice [38–40] there was no evidence of tumorigenesis. Additionally, liver tumors have not been observed in long-term monkey studies (James M. Wilson, University of Pennsylvania, personal communication) nor in canine [41,42] AAV-based liver-directed gene transfer studies.

With respect to their safety profile, AAV vectors have been injected in more than 300 subjects with no serious adverse events reported [11]. An exception was the death of a young woman in 2007 who was enrolled in a gene therapy clinical trial for rheumatoid arthritis [43]. In this trial, an AAV1 vector expressing a tumor necrosis factor alpha (TNF-α) inhibitor was injected intra-articularly to one knee of each enrolled subject. The female subject received the second vector injection 5 months following the first injection, however she died within 22 days of the injection [43]. Detailed postmortem examination of the subject and extensive review by an expert panel convened by the NIH Recombinant DNA Advisory Committee concluded that the death of the subject was not related to the AAV vector-infusion but rather due to a disseminated fungal infection [11].

Until 2002, there were just six AAV vector serotypes (AAVs 1–6), each with different tropism and transduction efficiency properties. Most preclinical and clinical studies focused on the prototype AAV serotype 2. Since 2002, the AAV vector toolkit has been significantly enriched with the isolation and characterization of novel serotypes that feature new tropism, improved transduction and safety

profiles as well as scalable manufacturing protocols for clinical trials [44–48]. One of these serotypes, AAV8 [44], discovered in the laboratory of James M. Wilson, has been extensively studied for liver-directed gene transfer and its clinical utility is currently being assessed in numerous preclinical and clinical studies for the treatment of various genetic diseases that include familial hypercholesterolemia [49,50], hemophilia B [51,52], ornithine transcarbamylase deficiency [53–56], and Duchenne muscular dystrophy (DMD) [57]. Currently, there are more than 80 clinical trials using various AAV vectors to treat specific genetic diseases and disorders. Importantly, efficacy at varying levels has been reported in gene therapy clinical trials for Leber congenital amaurosis (LCA) [58–61], Parkinson's disease [62–64], hemophilia B [65–67], and lipoprotein lipase (LPL) deficiency [11].

Retrovirus-based vectors

The retrovirus is an enveloped single-stranded RNA virus that belongs to the Retroviridae family. The Moloney murine leukemia retrovirus (gammaretrovirus) has been extensively studied as a vector that can package up to 8 kb, which can stably integrate its genome in dividing cells only. However, the integration is random and expression of the transgene is subject to silencing. The targeting efficiency of retroviral vectors can be altered or greatly improved by pseudotyping the vectors with glycoproteins from the envelope of viruses that target a specific cell type [68,69].

Although long-term gene expression as well as the generation of a population of gene-corrected cells is a desirable outcome for gene therapy, retrovirus-based vectors have been shown to be associated with a high probability of insertional mutagenesis due to random integration [70]. In ex vivo phase I clinical trials for X-linked SCID (SCID-X1) conducted in France [71] and the United Kingdom [72], bone marrow-derived CD34+ cells were harvested, transduced ex vivo with gammaretroviral vectors expressing the gamma chain and re-infused into nine infant/toddler-aged male subjects in France [71] and in 10 subjects in the United Kingdom [72]. The therapeutic outcomes were immediate and reconstitution of the immune system was observed in almost all subjects. However, in an unfortunate turn of events, 4 of the 9 subjects in the French trial [73,74] and 1 of the 10 subjects in the United Kingdom trial developed leukemia-like T lymphoproliferative disorder [70] linked to the retrovirus-mediated insertional mutagenesis. In combination, these serious adverse events prompted the voluntary hold of the trials.

Lentivirus-based vectors

Lentiviruses are a group of retroviruses that belong to the Retroviridae family. Unlike retrovirus-based vectors,

lentiviral (LV) vectors can transduce quiescent cells [75,76]. Considerable progress has been made in the development of LV vectors, especially those based on the human immunodeficiency virus type I (HIV-1) [75,76], the feline immunodeficiency virus (FIV) [77,78], and the equine infectious anemia virus (EIAV) [79]. These vectors have been disabled to render them safe for gene transfer *in vivo*. When developing LV vectors for application to specific tissues, their targeting efficiency is enhanced by optimizing the envelope pseudotype. Indeed, LV vectors pseudotyped with the envelope glycoproteins of the vesicular stomatitis virus [80], Mokola [81], Sendai [82], Ebola [83,84,81], baculovirus protein glycoprotein 64, severe acute respiratory syndrome [85], and avian influenza [86] viruses have been shown to result in improved transduction in various tissues including hematopoietic stem cells [87], lung [80–84,86] and eye [88,89]. Similar to AdV vectors, LV vectors have a large packaging capacity and like all retroviruses can stably integrate their genetic cargo into the genome of the host cell.

Although integration is random [90], LV vectors result in long-term transgene expression that is not subject to gene silencing mechanisms, in a variety of tissues that include brain [75], muscle [91], lung [80], liver [92], and eye [93]. Although LV vectors hold great promise as gene transfer vectors their origin, especially for those based on HIV-1, has naturally raised safety concerns. As such, great care has been taken to develop LV vector systems designed with safety features to prevent the emergence of replication competent lentivirus (RCL). Typically, the accessory genes (*vif*, *vpr*, *vpu*, and *nef*) and the regulatory genes (*tat* and *rev*) are separated from the *gag*, *pol*, and *env* genes [94], resulting in a theoretically negligible risk of emergence of an RCL that shares the pathogenic features of the parental virus. The safety of LV vectors has further been improved by the construction of self-inactivating (SIN) LV vectors [95].

Clinically, LV vectors have shown impressive efficacy. In 2009, an HIV-1-based SIN LV vector was used to transduce *ex vivo* CD34$^+$ cells isolated from two young male patients suffering from X-linked adrenoleukodystrophy (ALD), a demyelinating disorder of the central nervous system. The transduced hematopoietic stem cells were then re-infused into the subjects [87]. Disease progression in both subjects was halted and evidence of mild symptom reversal was observed [87]. Recently, in an *ex vivo* phase I clinical trial an SIN LV vector expressing anti-CD19 linked to CD3-zeta and CD137 signaling domains was used to transduce *ex vivo* T cells harvested from a subject with refractory chronic lymphocytic leukemia [96]. The vector-treated T cells were then re-infused into the subject and expanded to a level that resulted in complete remission. These impressive data are forming the basis for further larger clinical trials. Nonetheless, strict attention is

being paid to the risk of the potential of insertional mutagenesis for these LV vectors as well [96]. Specifically, in the ALD trial, investigators noted the presence of common insertion sites (CIS) in the treated cells [97]. Although these were found to be non-genotoxic CIS, the real risk needs to be addressed or at least carefully monitored in larger clinical trials using LV vectors.

Non-virus-based vectors

The efficiency of most non-viral gene transfer vectors (i.e. lipoplexes) used to deliver genes into target cells largely depends on the mechanisms that are present in the target cells for uptake and intracellular transport of particles [98]. Non-viral-based vector systems are not as efficient as viral-based vector systems for three main reasons: (a) limited ability of cationic lipids to bind to the surfaces of target cells, (b) low accumulation of cationic lipid/DNA complexes within the cell, and (c) ineffective translocation of DNA through the nuclear membrane to the nucleus [98]. Nonetheless, non-viral-based vectors are considered safe and less immunogenic than viral vectors [98]. Currently the only active clinical trial worldwide for CF airway disease, is one being conducted by the United Kingdom Gene Therapy Consortium, and uses GL67 [99] (a cationic lipid) to encapsulate the CFTR-DNA for delivery to the airway of CF patients 12 years and older. In an earlier clinical trial, it was demonstrated that delivery of nebulized GL67-CFTR into the lungs of CF subjects corrected the Cl$^-$ transport defect in the airways [100].

Although beyond the scope of this review, numerous other viruses are being developed as gene transfer tools, including, but not limited to, vaccinia virus [101], human parainfluenza virus [102,103], human respiratory syncytial virus [104], alphavirus [105], and herpes simplex virus [106], as well as physical (e.g. electroporation [107] and magnetofection [108]) and chemical (e.g. lipoplexes [108]) methods, including RNA nanotechnology [109], to improve vector (or DNA) delivery.

Modeling Gene Therapy in Animals

The most important aspect for the clinical translation of gene therapy vectors is the evaluation of vector efficiency and safety in relevant animal models. Vector-mediated correction of disease phenotype in the mouse model forms the basis for preclinical evaluation in larger animal models prior to clinical trials. In recent years, the challenges of translating findings from mouse studies to a higher animal species, or even humans, have become increasingly apparent. It is almost impossible to predict how a vector will behave in a higher animal species, let alone in a human subject. The reasons for the differences in gene transfer efficiency or heightened immune responses are the species-

specific differences in vector targeting, uptake and processing in target cells, as well as the complex and sophisticated immune system of higher species.

One excellent example of the discrepancy in the efficiency and safety observed between animal models and human subjects was the recent hemophilia B trial using an AAV2 vector [66]. Long-term gene transfer studies in large canines [110,42], and non-human primates [111,112] predicted that a therapeutic dose (2×10^{12} vector genomes/kg) of AAV2 would be effective at restoring adequate levels of serum-circulating levels of factor IX (FIX). However, following a liver-directed injection of AAV2-FIX vector in adult subjects with hemophilia B, only transient expression of FIX was observed [66]. The reasons for this apparent discrepancy are not entirely understood. However, it has been postulated that memory T cells against the AAV2 capsid, in combination with the compromised liver microenvironment of most patients with hemophilia B that have become infected with hepatitis B and C, contributed to the unpredicted cytolytic capsid-directed T cell response [113]. Current clinical trials for hemophilia B are employing two differing strategies: AAV2 with short-term immunosuppression and self-complementary AAV8 (at lower vector doses) in the absence of immunosuppression [11]. In addition to AAV2, AAV1 has also shown to be immunogenic when injected intramuscularly in humans. Specifically in a clinical trial for LPL deficiency, subjects were injected with AAV1 intramuscularly and at the higher vector doses capsid-specific CD4$^+$ and CD8$^+$ T cells were detected [11]. In another clinical trial using AAV1 to express α1-antitrypsin (A1AT) in subjects with A1AT deficiency, T cell activation against the capsid following intramuscular injection of vector was also noted [114].

In addition to humoral and cellular responses to the viral capsid [115,11], gene therapy carries the risk of activating the immune system to the wild-type version of the missing or mutated protein [114,116]. Significant frequencies of circulating transgene-specific T cells have been reported in subjects treated with AAV vectors for various diseases including A1AT deficiency [114] and DMD [116]. A recent report of gene replacement therapy for DMD suggests that some subjects may be at increased risk of clinically meaningful cytotoxic T cell responses against the transgene-derived dystrophin protein due to pre-existing memory T cells to dystrophin. The authors concluded that primed T cells may contribute to DMD pathogenesis and enhance problematic T cell responses following gene therapy that aims to reconstitute normal dystrophin expression [116]. More concerning, however, for the application of gene therapy vectors in human subjects may be the prevalence of pre-existing T cells against the therapeutic gene reported for dystrophin [116] and recently CFTR [117]. The reason why these pre-existing T cells are present in patients with a specific genetic disease remains to be elucidated. Nonetheless, the role of these resident transgene-specific T cells in disease pathogenesis or the possible increased likelihood of rejection of transduced target cells following gene therapy needs to be carefully investigated. Predicting T cell activation in humans following gene therapy is considered a top priority. Prior exposure to an AAV or another virus and/or the compromised or heightened immune system as a result of the specific disease phenotype may contribute to the likelihood of T cell activation following gene therapy but this remains to be proven.

Despite the limitations of translational liver- and muscle-directed gene therapy, two examples in which animal studies accurately predicted the vector safety and the efficacy were apparent in the clinical trials for LCA [58–61]. The dose, safety profile, and therapeutic outcomes of the AAV2 vector in young and adult human subjects successfully mirrored findings from small and large animal studies [58–61].

Gene Therapy: Early Disease Treatment

The majority of patients who suffer from the most common genetic diseases are diagnosed prenatally if there is a family history or soon after birth as part of standardized postnatal screening that includes CF, blood cell disorders (β-thalassemia), inborn errors of (i) amino acid metabolism (e.g. phenylketonuria), (ii) organic acid metabolism, and (iii) fatty acid metabolism. However, most gene therapy clinical trials focus on treating adult subjects in which disease progression has caused irreversible damage to the target tissues. Although there continues to be strong ethical debates regarding gene therapy in fetuses and/or young children, significant progress in pre- and postnatal gene therapy in various animal species, including macaques, have demonstrated both safety and efficacy. Specifically, exciting data from AAV gene transfer studies conducted *in utero* or shortly after birth in large animal models (macaques) now pave the way for prenatal gene therapy for genetic diseases with acute and immediate life-shortening symptoms. Two examples are ornithine transcarbamylase deficiency [118], which causes irreversible damage in young male babies soon after birth with 50% of subjects dying within weeks of birth [119,120], and some types of mucopolysaccharidoses in which irreversible brain damage occurs prior to or soon after birth [121]. For these young subjects, the therapeutic benefit of gene transfer prior (prenatal) or immediately following (postnatal) birth would be immediate, allowing for prolonged life and improved quality of life.

Gene therapy in fetuses and babies diagnosed with genetic diseases such as CF and DMD is also warranted. The level of damage in the affected tissue at birth is minimal, but the benefit of early therapeutic gene

expression in the target tissue is maximal. The logistical limitation of vector dose needed for injection is minimized given the small size of a baby (∼3 kg). Although there is debate surrounding the impact of serum-circulating neutralizing antibodies in babies, recent data generated in macaques demonstrate that maternal AAV-specific antibodies passed to the baby *via* the umbilical cord can cross-neutralize the vector and decrease the efficiency of gene transfer [54]. Nonetheless, prenatal gene therapy takes advantage of the immature immune system that remains ignorant to the expression of a 'foreign' antigen. This allows for the induction of tolerance against therapeutic proteins and long-term gene expression. Continued work in prenatal and postnatal gene transfer with AAV [122] and lentiviral [123] vectors is focused on improved gene transfer, increased safety and reducing the risk of germ line transmission.

Gene Therapy: Back in the Limelight

The true potential of gene therapy was realized recently in patients suffering from an inherited recessive disease of blindness, LCA [58–61]. Mutations in thirteen different genes have been associated with LCA with mutations in the retina pigment epithelium-specific 65-kDa protein (RPE65) accounting for ∼10% of LCA sufferers [124]. AAV2-mediated restoration of vision in mouse and dog models formed the basis of three separate clinical trials in the United States and the United Kingdom [58–61] aimed at restoring vision in subjects with LCA as a result of the RPE65 mutation. Improvement in visual function was evident within weeks of vector injection and remarkable results of vision restoration in young subjects re-established the hope and increased expectations for what gene therapy can realistically deliver. AAV vectors and the plethora of serotypes that are currently available have positioned AAV as a key player in virus-based gene therapy clinical trials. Indeed the majority of currently active clinical trials make use of AAV vectors. Specifically, AAV8 vectors are in the clinic for gene therapy of hemophilia B; AAV1 vectors for A1AT deficiency, muscular dystrophy Types 2C and 2D, LPL deficiency, Pompe disease, and severe heart failure; AAVrh10 for Batten's disease and Sanfilippo syndrome type A; and AAV2 for hemophilia B, LCA, age-related macular degeneration, Alzheimer's disease, Canavan's disease, and Parkinson's disease. An expanded list of active clinical trials using gene transfer vectors can be found at http://clinicaltrials.gov or for trials conducted in Europe or Australia at https://www.clinicaltrialsregister.eu or http://www.abedia.com/wiley.

With the completion of the human genome project came much speculation about the innumerable prospective uses of the newly discovered genetic information. Genetic predispositions for acquired diseases will allow the development of aggressive and early interventions to prevent morbidity and mortality associated with a particular disease. In this vein, the greatest potential of the sequenced human genome, coupled with the availability of highly efficient vectors, is personalized medicine for acquired (e.g. cancers) or genetically predispositioned (e.g. diabetes and heart disease) conditions.

The recent successes of gene therapy in clinical trials are long overdue and have served to refocus attention and build palpating excitement around its incredible promise. There are multiple clinical trials in the United States and Europe that are currently recruiting subjects for genetic diseases, including DMD, hemophilia B, and for diseases affecting vision. Gene therapy is ready for primetime and the prediction for the next few years is that the field will witness many more clinical successes.

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